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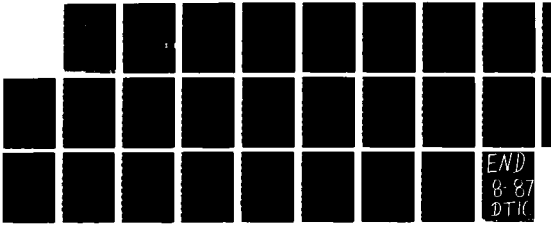

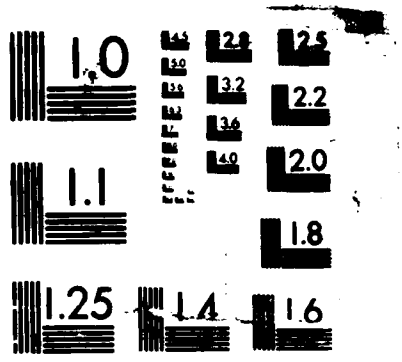

MICROCOPY RESOLUTION TEST CHART

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NEUROPEPTIDES IN EXPERIMENTAL HEAD INJURY

ANNUAL REPORT

ALAN I. FADEN
TRACY K. MCINTOSH

FEBRUARY 28, 1986

Supported by
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Much of the damage resulting from ischemic or traumatic insults to the central nervous system appears to result from secondary injury mechanisms relating to the release of endogenous factors. Endogenous opioids may represent one such class of pathophysiological factors, and have been implicated in traumatic spinal cord injury, ischemic spinal cord injury and ischemic brain injury. The studies covered under the present contract examine the potential pathophysiological role of endogenous opioids and their receptor-mediated changes following traumatic brain injury in both cats and rats. Utilizing a fluid

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FOREWARD

Due to a moratorium placed on U.S. Army Medical Research Contracts pertaining to the utilization of cats in experimental research, the studies described herein represent work performed over an 8-month period. In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

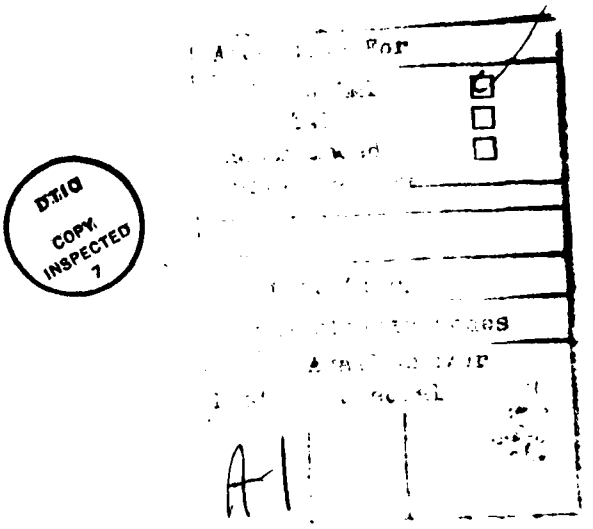

TABLE OF CONTENTS

SUMMARY

Much of the damage resulting from ischemic or traumatic insults to the central nervous system (CNS) appears to result from secondary injury mechanisms relating to the release of endogenous factors. Endogenous opioids may represent one such class of pathophysiological factors, and have been implicated in traumatic spinal cord injury, ischemic spinal cord injury and ischemic brain injury. The studies covered under the present contract examine the potential pathophysiological role of endogenous opioids and their receptor-mediated changes following traumatic brain injury in both cats and rats. Utilizing a fluid-percussion device manufactured for us by Medical College of Virginia, we have evaluated the effects of graded levels of injury on outcome measures, including mean arterial pressure, intracranial pressure, electroencephalographic (EEG) activity (including the Fast-Fourier transformed EEG), and regional cerebral blood flow in the cat. In studies conducted during the present Army contract we have compared the effectiveness of the opiate antagonist WIN44,441-3, which has enhanced activity at the κ-opiate receptor, with its inactive stereoisomer WIN44,441-2 and saline.

Following a brief period of hypertension (1 - 3 minutes), fluid-percussion (3.0 atmospheres [atm]) in the cat produces a significant decrease in mean arterial pressure by 1 hour postinjury. Whole brain blood flow (measured using the radiolabeled microsphere technique) was also significantly reduced ($p < 0.05$) when measured at 2 hours following injury. Regional blood flow also fell significantly in frontal cortex ($p < 0.05$), striatum ($p < 0.05$), midbrain ($p < 0.05$) and brainstem ($p < 0.05$) at 2 hours postinjury. Fluid-percussion injury also causes a concomitant decline in spectral edge and compressed spectral array EEG with a significant decrease in EEG amplitude (as expressed by power band analysis, $p < 0.05$). Administration of the opiate

receptor antagonist WIN44,441-3 (which has enhanced activity at κ-receptors) at 15 minutes following head injury (n = 6 cats) caused a rapid and significant increase in mean arterial pressure that was sustained for up to 2 hours, with an associated increase in whole brain blood flow. Blood flow was also significantly increased in the midbrain, brainstem, cerebellum and basal ganglia where injury, evidenced by histological changes, was most severe. Administration of WIN44,441-3 also caused a significant improvement in amplitude of EEG power bands and a return to baseline values of the spectral edge. This improvement in EEG paralleled the improvement in mean arterial pressure that occurred following administration of the κ-opiate receptor antagonist. Administration of the inactive stereoisomer (n = 6) or saline (n = 6) had no effect on cardiovascular or electrophysiological function.

Preliminary work in our laboratory with a rat model of lateralized head injury has supported our results obtained using the cat. Traumatic brain injury in the rat produces a series of physiological responses identical to those observed in the cat (hypotension, fall in EEG amplitude, shift in EEG frequency). We have observed that animals treated with nalmefene, a newly synthesized opiate antagonist with increased affinity at κ-receptors, demonstrate a recovery of mean arterial pressure and EEG parameters. Together, these results suggest that endogenous opioids contribute to the pathophysiology of head injury and indicate that opiate antagonists with increased activity at κ-sites may be effective in the treatment of low flow states associated with acute head injury.

STATEMENT OF PROBLEM

It is clear that much of the neurological deficit which follows traumatic injury to the CNS results not only from the immediate and direct effects of the trauma in severing neuronal connections, but from secondary responses initiated

by the injury that results, in part, from reductions of blood flow due to release endogenous factors. It has previously been established that endogenous opioid systems contribute to the secondary pathophysiological events in spinal cord injury by contributing to the reduction in spinal cord blood flow (SCBF). Although work in our laboratory has suggested that endogenous opioids, acting at κ-receptors in the spinal cord, may act as pathophysiological factors in traumatic spinal cord injury, the response of physiologically active endogenous opioid systems to traumatic brain injury is unknown, and the possible role of endogenous opioid systems in the pathophysiology of traumatic brain injury is essentially unexplored. Such studies may lead to a better understanding of the secondary pathophysiological changes that occur after traumatic brain injury and may lead to novel pharmacological therapies such as selective opiate-receptor antagonists (e.g., κ-receptor antagonists) or physiological opiate antagonists (e.g., TRH, TRH analogs).

BACKGROUND

It has been suggested that the endogenous opioids may contribute to CNS injury, in part, by reducing blood flow to the CNS or by altering brain metabolic activity (1). Based on our work in experimental shock, we have previously demonstrated that traumatic cervical spinal cord injury in the cat caused an elevation of plasma β-endorphin-like immunoreactivity associated with reduction in SCBF (2,3). Treatment with high doses of naloxone (2 mg/kg) in these studies significantly improved both SCBF and neurological recovery. This beneficial effect of high-dose naloxone in spinal cord injury has been confirmed by other researchers (4-6). Since it is known that δ- and κ-receptors are less naloxone sensitive (7), the high pharmacological doses of naloxone required in the spinal injury studies suggest that the naloxone effects may have been due to actions at

such non-μ opiate receptors. To this end, we have demonstrated that the dynorphin family of opioids is unique in producing dose-related hindlimb paralysis following intrathecal administration in rats (8). Since dynorphin is proposed as the endogenous ligand for the κ-receptor, this observation suggested that the dynorphin/κ-receptor system may be involved in the pathophysiology of spinal cord injury. Dynorphin A-immunoreactive material (Dyn A-ir) (1-17), but not leu-enkephalin, met-enkephalin or Dyn A (1-8), was found to be increased at the injury site following experimental traumatic spinal cord injury in the rat (9). It has also been observed that the opiate antagonist WIN44,441-3, which has increased activity at κ-sites, stereospecifically improves neurological recovery after traumatic spinal injury in the cat ischemic spinal injury in the rabbit (10). Since WIN44,441-3 was approximately 50 times more potent than naloxone in its therapeutic effects in the rabbit, the relative dose-response effects are consistent with a κ-receptor mechanism of action (11).

Although the physiological changes following head injury that have been related to clinical outcome include changes in blood pressure, hypoxia and ischemia (12-14), little is known concerning the role of endogenous opioids in mediating the pathophysiological sequelae of head trauma. Naloxone treatment, at high doses (2 mg/kg), has been found to significantly improve the cortical somatosensory-evoked response and prevented the areas of multifocal low blood flow and infarction following ischemic brain injury produced by air embolism in the dog (15). Hayes et al. recently demonstrated that high doses of naloxone treatment improved blood pressure, brain perfusion pressure and the cortical electroencephalogram following concussive brain injury in cats (16). Although these authors did not measure plasma or regional brain opioid peptides, their data are the first to suggest that endogenous opioid substances may be released

after experimental closed head injury. Studies from our laboratory, supported by the present contract, have recently demonstrated that the κ-selective opiate antagonist WIN44,441-3 stereospecifically improves blood pressure and focal cerebral blood flow following closed head injury (17). No studies to date, however, have examined the role of specific endogenous opioid systems, particularly dynorphin, in mediating the cardiovascular changes following head trauma.

Thyrotropin-releasing hormone (TRH) has been tested in spinal cord injury for the same reasons it was evaluated in experimental shock, based on its ability to act in vivo as a partial physiological antagonist of endorphin systems. Not only was TRH found to be beneficial in treating traumatic spinal cord injury in the cat, but TRH proved significantly superior to both naloxone and high-dose corticosteroids in this regard (18). The beneficial effect of TRH on motor recovery following traumatic cervical injury in the cat was dose related, with significant actions noted at doses as low as 0.02 mg/kg (as IV bolus plus 4-hour IV infusion; total dose, 0.1 mg/kg). In addition, TRH proved to be effective, even when treatment was delayed as long as 24 hours after injury (18).

Given the obvious similarities between pathophysiological consequences of head injury and spinal cord injury, it is somewhat surprising that so little experimental work in head injury has been performed to date. In a single report, TRH treatment has been found to improve neurological function and EEG following brainstem compression in the cat (19). This study and the results obtained from spinal cord injury studies suggest a potential therapeutic role for TRH in traumatic brain injury.

APPROACH TO PROBLEM

Our technical objectives under the current contract are: (a) to evaluate changes in endogenous opioids after traumatic brain injury in the cat and the

rat; (b) to characterize the physiological response to graded levels of traumatic brain injury, including mean arterial pressure, intracranial pressure, brain perfusion pressure, cerebral blood flow, electroencephalographic changes, and brainstem auditory evoked responses; (c) to examine the therapeutic effects of selective κ-opiate receptor antagonists and analogues after experimental head injury; (d) to determine whether more selective opiate antagonists or TRH analogs are more effective than naloxone in the treatment of closed head injury; and (e) to compare the effects of the various pharmacological treatments on blood flow and physiological changes following traumatic brain injury.

Brain injury was induced by a fluid-percussion device that causes graded brain injury through brief distortion of neural tissue (20). Eighteen male or female cats (3.0 - 3.5 kg) were anesthetized with sodium pentobarbital (50 mg/kg), paralyzed with a continuous infusion of pancuronium (0.6 mg/kg/hour) and artificially ventilated throughout the experiment with 70% nitrous oxide and 30% oxygen using an Omni Veterinarian Anesthesia Machine (Ohmeda Corporation, San Rafael, CA) and a Harvard ventilator (Harvard Apparatus, Milton, MA). Drugs were administered through a cannula placed in the inferior vena cava via the femoral vein. The femoral artery was cannulated (PE90) to monitor heart rate, mean arterial pressure (MAP) and pulse pressure (PuP) as well as to sample arterial blood gases. Prior to injury, sodium bicarbonate (0.16 mEq/ml) was administered as required to maintain pH within the normal range. With the animal in a stereotaxic frame, the scalp and temporal muscle were reflected and a hollow 17.5 mm "trauma" tube rigidly fixed with dental acrylic to the animal's skull over an 18.0 mm craniotomy centered over the sagittal sinus, midway between lambda and bregma.

Mechanical deformation of the brain was produced by a fluid-percussion device, initially developed as a clinically relevant animal model of brain concussion (20,21). This device is refinement of earlier fluid-percussion head injury models developed in other laboratories (21) and produces graded levels of brain injury associated with sudden deformation of neural tissues. Brain deformation results from the introduction of small volumes of fluid into the epidural space of the closed cranium by a metal pendulum which strikes the piston of the device from a predetermined height, forcing a fixed volume of isotonic saline through the hollow "trauma" tube into the skull cavity. The device produces a pulse of increased intracranial pressure (ICP) of fairly constant duration (21 - 23 msec). Since increasing fluid loading produces greater magnitudes of injury, the magnitude of injury is regulated by varying the height of the pendulum.

A piezoelectric pressure transducer affixed to the injury device measured the pressure transient which was recorded on a storage oscilloscope (Tektronix) and photographed with a Polaroid camera. The duration and peak pressure in atmospheres (atm) was noted for each injury.

Intracranial Pressure and Mean Arterial Blood Pressure:

A small hole (7.0 mm in diameter) was drilled in the skull, and the dura was exposed in order to place a plastic cannula to record intracranial pressure (ICP). The exposed dura was cut so that the cerebrospinal fluid had unobstructed access to the cannula. The hole was sealed with dental acrylic, and the cannula was filled with normal saline. This method of monitoring ICP has been used successfully in many previous studies of fluid-percussion injury (21) since insertion of an intracerebroventricular cannula can cause additional tissue injury when fluid-percussion is initiated. Changes in MAP and ICP were monitored by strain gauge transducers, the outputs of which were recorded on a

Narco Biosystem NT-40 polygraph (Narco Corporation, Houston, Texas). Arterial blood gases were analyzed on an IL Model 213 pH and blood gas analyzer (Instrumentation Laboratories, Lexington, MA).

Electroencephalography (EEG):

During surgery, aluminum screws (2.56 mm x 3/8) were placed over the left and right parietal cortices for EEG recording. Fast-Fourier transformed computerized electroencephalogram (FFT-EEG) were recorded continuously on a Neurotrac Computerized EEG (Interspec, Inc., Philadelphia, PA). Left and right "raw" EEG, compressed spectral array (CSA), spectral edge (the EEG frequency below which 95% of all EEG activity occurs) and EEG amplitude (as measured by power band analysis) were continuously recorded over the duration of the study. EEG amplitude was measured in picowatts with multiple filter range set at 160 microvolts over 4 second epochs. Compressed spectral array was measured over 4 second epochs with sensitivity set at 1 to 30 Hz (spectral edge was set at 95% of total power).

Cerebral Blood Flow:

The radioactive microsphere technique was used to measure CBF because the technique allows for repeated measurements of regional CBF with the same animal (22). The radionuclides used for these studies were niobium-95, gadolinium-153, strontium-85, scandium-46, and tin-113; all had a specific activity of 10 mCi/g. ^{85}Sr, ^{46}Sc, ^{95}Nb, ^{153}Gd, were obtained from 3M, New Brighton, Minnesota, and ^{113}Sn from New England Nuclear, Boston, Massachusetts. Experiments were carried out on 9 cats, anesthetized as previously described. A cannula (PE90) were also placed in the left femoral artery for withdrawal of reference arterial samples. A PE90 cannula with a slightly flared end was placed in the left atrium via a thoracotomy (see reference 6), and the chest was sutured closed.

For each CBF determination, 0.9 to 1.8 x 10^6 microspheres (15 μm in diameter) in dextran and polyethylene sorbitan mono-oleate (Tween 80) were injected at 5 time points: prior to injury (baseline), 10 minutes postinjury (prior to drug administration), 30 minutes, 1 h and 2 h after injury. Following agitation for 4 minutes on a vortex mixer, microspheres were injected into the left atrium over approximately 30 seconds. The injection of this number of microspheres insured that tissue samples over 250 mg would contain at least 400 microspheres (23). Just before and for 90 seconds after each microsphere injection, reference arterial samples were withdrawn from the left femoral artery at a rate of 0.6 ml/minute using Gastight syringes (Hamilton Company, Reno, Nevada) and a Harvard withdrawal pump (Harvard Apparatus, South Natick, Massachusetts). Counts from the arterial reference samples were used to calculate CBF.

After the final microsphere injection the animals were killed by transaortic perfusion with 0.9% NaCl solution followed by aldehyde fixatives. The brains were removed, sectioned coronally, and the frontal cortex, striatum, hippocampi, diencephalon, parietal cortex, midbrain/thalamus were dissected bilaterally. Pons, medulla and cerebellum were also dissected. This procedure yielded tissue samples varying in weight from 250 mg to 1.5 g. Tissue samples were weighed and counted, along with the arterial reference samples in a Beckman Gamma 300 gamma counter (Beckman Instruments). Matrix inversion and CBF calculations were performed using a Datapoint 1800 minicomputer. Cerebral blood flow was calculated using the following equation (22,23):

$$\text{CBF (ML/100 G/MIN)} = \frac{Cb \times 100 \times RBF}{cR},$$

where cB indicated counts in brain, Cr indicates counts in arterial samples, and RBF is the arterial withdrawal rate.

Tissue Pathology:

Upon brain dissection for peptide analysis or regional CBF studies, the occurrence of regional tissue hemorrhage and/or necrosis were recorded for animals treated with WIN(-) (n = 6), WIN(+) (n = 6) or saline (n = 6). Scoring for postinjury pathological tissue damage was performed in a blinded fashion, and was based on the following scale: 0 = no observable damage; 1 = slight hemorrhage present; 2 = moderate hemorrhage present; 3 = severe hemorrhage and/or necrosis present.

Experimental Protocol:

In order to examine the role of specific endogenous opioid receptors in traumatic brain injury, 15 minutes following brain injury of similar magnitude (3.2 - 3.6 atm), one group of animals received either: (1) the opiate antagonist WIN44,441-3 [WIN(-)] which has increased activity at κ-sites (24) (0.2 mg/kg in 10 cc saline, n = 6); (2) its dextrostereoisomer WIN44,441-2 [WIN(+)], which is inactive at opiate receptors (0.2 mg/kg in 10 cc saline, n = 6); or (3) saline vehicle (10 cc, n = 6) in a double-blind fashion. The dose chosen was based upon previous work in our laboratory relating to spinal trauma and ischemia (16). Regional cerebral blood flow (CBF) was measured in a subgroup of animals using the radiolabelled microsphere technique as described above. Sequential measurements of CBF were performed 10 minutes prior to injury (baseline) and 10, 30, 60 and 120 minutes after injury in animals receiving WIN(-) (n = 3), WIN(+) (n = 3) or saline (n = 3).

Data Analysis:

All data are expressed as mean ± S.E.M. Statistical analyses were performed employing parametric analyses of variance (ANOVA) for repeated measured

followed by Duncan's multiple range test. For cerebral blood flow, statistical comparisons were carried out using analysis of covariance and Duncan's multiple range test. Kruskal-Wallis ANOVA was used to compare all ordinal data. Fisher's Exact Probability Test was used to compare survival data. A 'p' value < 0.05 was considered statistically significant.

RESULTS

MAP and ICP:

Mean arterial pressure in all animals increased approximately 90 mm Hg (from 135 ± 6 to 220 ± 5 mm Hg) by 2 minutes postinjury and remained elevated for up to 5 minutes. By 15 minutes postinjury (immediately prior to drug administration), MAP had returned to control values. Subsequently, MAP in all saline- and WIN(+)-treated animals continued to fall, reaching hypotensive levels (as compared to baseline: MAP $\leq$ 90 mm Hg) by 2 hours postinjury). Administration of WIN(-) at 15 minutes following injury reversed the fall in MAP within 5 minutes. WIN(-)-treated animals had significantly elevated when compared to WIN(+)- or saline-treated animals ($p < 0.01$) over the remainder of the 2-hour monitoring period. A transient increase in ICP to 47 ± 5 mm Hg was observed immediately following injury, but ICP returned to normal by 10 minutes postinjury. Administration of WIN(-), WIN(+) or saline had no effect on posttraumatic intracranial pressure.

EEG:

Fluid-percussion injury caused a rapid fall in EEG amplitude, as measured by power band analysis, to 36 ± 5% of control levels ($p < 0.001$) by 1 minutes postinjury and to 32% ($p < 0.001$) of control by the end of the 2-hour study period in WIN(+) or saline-treated animals. In over 40% of all animals following injury, the diminution of EEG amplitude was so marked following injury that the CSA spectral edge was entirely abolished.

Administration of WIN(-) caused a significant increase in EEG amplitude within 5 minutes of drug treatment ($p < 0.001$). By 15 minutes after drug treatment, EEG amplitude had returned to 78% of control values; this increase was maintained throughout the remainder of the 2-hour observation period. Following administration of WIN(-), the significant increase in EEG amplitude was also accompanied by a restoration of the EEG waveform and spectral edge. Administration of WIN(+) was without effect on restoring the spectral edge in those animals.

Regional CBF:

Traumatic brain injury in saline-treated animals caused either a slight increase or no change in regional CBF by 10 minutes postinjury. Regional CBF began to decline by 30 minutes postinjury and at 2 hours postinjury was significantly depressed in the frontal cortex (-29%; $p < 0.05$), striatum (-40%; $p < 0.05$), hippocampus (-52%; $p < 0.05$), midbrain/thalamus (-42%; $p < 0.05$), pons (-39%; $p < 0.05$) and medulla (-46%; $p < 0.05$). A significant decrease in whole brain blood flow was also observed in saline-treated animals at 2 hours postinjury (from 40 ± 7 baseline to 32 ± 5 ml/100 g/minute, $p < 0.05$).

In all animals treated with WIN(+) 15 minutes after injury, whole brain blood flow continued to decline after injury and was significantly depressed at 2 hours postinjury ($\bar{x}$ = 31 ± 4 ml/100 g/minute, $p < 0.05$). Regional CBF declined maximally in WIN(+)-treated animals at 2 hours postinjury in those areas demonstrating greatest pathological changes on gross examination: striatum (-27%; $p < 0.05$); midbrain (-40%; $p < 0.05$); frontal cortex (-28%; $p < 0.05$); pons (-34%; p = nonsignificant); medulla (-29%; $p < 0.05$); and cerebellum (-44%; $p < 0.05$).

Administration of WIN(-) at 15 minutes postinjury caused a significant increase in whole brain blood flow at 2 hours postinjury (from 40 ± 5 to 53 ± 6

ml/100 g/minute; $p < 0.05$). Regional blood flow measured at 2 hours postinjury was also significantly increased by WIN(-) treatment when compared to postinjury values; frontal cortex (+44%; $p < 0.01$), striatum (+36%; $p < 0.05$), midbrain (+30%; $p < 0.05$), pons (+25%; p = nonsignificant).

Survival:

Among control animals, 3 of 6 animals treated with WIN(+) and 2 of 6 animals treated with saline died within the 2-hour study period after injury. All saline- and WIN(+)-treated animals died when they became severely hypotensive. In contrast, all 6 animals receiving WIN(-) survived following trauma. This difference in survival between WIN(-) and control [saline- or WIN(+)-treated] animals was statistically significant ($p = 0.04$, Fisher's Exact Probability Test).

Tissue Pathology:

Upon brain dissection, it was observed that fluid-percussion injury in saline-treated animals caused reproducible intraparenchymal hemorrhage in the midventral aspect of the pontomedullary junction (100% of animals), frontal cortex (80% of animals), striatum (80% of animals), midbrain/thalamus (100% of animals), hippocampus (50% of animals), hypothalamus (80% of animals), pons (100% of animals) and medulla (100% of animals). This pattern and severity of intraparenchymal hemorrhage was similar to that observed in animals treated with WIN(+). However, in the WIN(-)-treated animals, the severity and incidence of hemorrhage was reduced in frontal cortex (65% of animals), midbrain/thalamus (50% of animals), hippocampus (25% of animals), and hypothalamus (25% of animals). The severity of hemorrhage was also found to be reduced in pons and medulla of WIN(-)-treated animals.

RAT STUDIES

During this contract we have successfully established a new traumatic head injury model in the rat, based upon the same fluid-percussion technology we have used in the cat. A reproducible injury curve has been generated in our laboratory, based on 24 hour postinjury neurological scores. These neurological scoring tests, which have been developed in our laboratory, appear to be highly sensitive to small changes in the level of severity of trauma. In contrast to the cat model, we are utilizing a lateralized head injury in rats, a model that is more cost effective and may be of more clinical relevance since it may produce a greater amount of cortical damage (shearing) than the traditional midline (vertex) model. Since preliminary studies (n = 20 animals) in our laboratory have indicated that the use of somatosensory-evoked responses are not quantifiably related to the severity of injury and do not appear to be predictive of posttraumatic neurological dysfunction, we have utilized the newly developed Siegen Neuroscope evoked potential recorder to analyze brainstem auditory evoked potentials (BAERs) following head injury in rats and subsequent treatment with opiate antagonists. Preliminary data suggests that changes in BAERs may have predictive value with regard to neurological dysfunction following traumatic brain injury. Finally, we have utilized the rat model to begin to examine the therapeutic efficacy of nalmefene, a newly synthesized opiate antagonist with increased affinity at κ-receptors in the treatment of lateralized fluid-percussion head injury. This drug was substituted for WIN(-) in these studies because nalmefene has a more highly selective profile at κ-opiate receptors and a longer duration of action. Several pilot animals, treated with nalmefene (0.1 mg/kg), have demonstrated a recovery of electroencephalographic activity and neurological function.

Surgical Preparation:

Forty-six male, Sprague-Dawley rats weighing from 400 - 500 g were initially anesthetized with ketamine (80 mg/kg, i.m.) and sodium pentobarbital (20 mg/kg, i.p.). During surgical preparation and throughout the experiment, all wounds were infused with a topical anesthetic (lidocaine hydrochloride, 2.0%). A bilateral femoral cutdown was performed and femoral venous (for drug administration) and arterial (for blood pressure/blood gas monitoring) catheters were inserted. With the animal in a stereotaxic frame, the scalp and temporal muscle were reflected. Next, a 2.0 mm hollow female Leur-Loc fitting (used to induce trauma) was rigidly fixed with dental cement to the animal's skull in a craniectomy centered over the left parietal cortex 5 mm from lambda, 5 mm bregma, 4 mm from sagittal suture; the dura was left intact at this opening. Stainless steel screw electrodes were inserted into the skull over the right sensory motor cortex (recording electrode) and the anterior nasal bone (reference electrode) to record brainstem auditory evoked potentials (BAERs) and EEG tracings. Immediately following surgical preparation, a constant i.v. infusion of sodium pentobarbital (15/mg/kg/hour) was begun and maintained for the duration of the physiological studies.

Fluid-percussion Injury:

The fluid-percussion device used to produce experimental brain injury was identical to that which has been used in cats and described previously.

Physiological Evaluation:

Recording screws for EEG were placed over the right and left parietal regions. The EEG electrodes were connected to a Neurotrac Systems Computerized spectral EEG analyzer (Interspec, Philadelphia, PA) in order to obtain continuous pre- and postinjury Fast-Fourier-transformed (FFT) spectral EEG recordings

including Fourier-transformed compressed spectral array (CSA), spectral histogram in the delta (0 - 4 Hz), theta (4 - 8 Hz), alpha (8 - 13 Hz) and beta (13 - 30 Hz) range, and frequency/amplitude (power band) analysis. To monitor BAERs, subdermal platinum needle electrodes were placed over the cortex (recording site) and in both ears. Stimulation of left and right eighth cranial nerve was accomplished using a manual click/tone stimulator (produced by a shielded Beyer transducer, energized by 100 μsec square wave pulse and presented at 25/second). Changes in function of the brainstem auditory pathway were examined on a Siegen Computerized Neuroscope (Siegen Incorporated, Palo Alto, CA).

Systolic, diastolic and mean arterial pressure (MAP) were recorded continuously before and after head injury via the femoral artery catheter. Pressures were monitored by strain gauge transducers, the output of which were recorded on a Narco Biotrace-40 physiograph (Narco Biosystems, Columbus). Arterial blood gases (pH, pO_2, pCO_2) were analyzed at regular intervals throughout the experiment using an Instrumentation Laboratories pH and blood analyzer (Instrumentation Laboratories, Braintree, MA).

Neurological Evaluation:

Chronic neurological scoring of motor function was performed daily by a trained observer who was unaware of each animal's level of injury using an ordinal scale. Animals were scored at 8 and 24 hours postinjury, and daily for 4 weeks after trauma. Animals were scored on a 4 (normal) to 0 (severely impaired) scale using each of the following indices: (a) forelimb flexion upon suspension by the tail; (b) decreased resistance to lateral pulsion; (c) circling behavior upon spontaneous ambulation; and (d) ability to stand on inclined angle board with the maximal angle at which the animal can stand for 5 second recorded (angle board) where 45 - 50° = 4, 40 - 45° = 3, 35 - 40° = 2,

30 - 35° = 1, 0 - 29° = 0. This ordinal grading scheme was very consistent among observers, with inter-rater reliability exceeding 93%. A total composite functional neurologic score (0 - 20) was obtained by combining the scores for the several tests of motor function so that 20 = normal; 15 = slightly impaired; 10 = moderately impaired; 5 = severely impaired; 0 = afunctional.

Histopathological Evaluations:

At 24 hours after low (Group I) or moderate (Group II) fluid-percussion injury animals (n = 3) were anesthetized and transcardially perfused with 10% buffered neutral formalin. The brains were removed, placed in fixative for a minimum of 1 week, and serial, coronal sections were cut (50 - 100 μm thickness) with a vibratome, beginning at the level of the optic decussation and extending to the mid-pontine region. Sections were mounted on gelatin-coated slides, hydrated, and stained with either hematoxylin and eosin or toluidine blue. After staining the tissue was dehydrated in graded alcohols and cleared in xylene for visualization at the light microscopic level.

In order to examine the relationship between magnitude of injury, protein extravasation and hemorrhage, 9 animals were injected intravenously with Evans Blue (25 mg/2.5% aqueous solution) at 5 hours following a low (Group I; n = 3), moderate (Group II; n = 3) or high (Group III; n = 3) injury. One hour later (t = 6 hours), the animals were sacrificed and perfused with fixative as described above. The brains were removed and photographed, then grossly dissected in order to define the regions of maximal hemorrhage and leakage to the Evans Blue Albumin complex. Control animals (n = 3) were prepared in a similar manner, including the administration of Evans Blue, but in the absence of injury.

Experimental Protocol:

During a 2-hour baseline period, MAP, EEG, and BAERs were continuously recorded and arterial blood gases monitored. At the end of the 2-hour baseline

period, animals were attached to the fluid-percussion device and injured at three levels of injury (low-grade, 0 - 1 atm = Group I; moderate, 1.5 - 2.0 atm = Group II; high-grade, 2.5 - 3.6 atm = Group III, n = 8/group). MAP, EEG, BAERs, and ABGs were monitored continuously for 2 hours postinjury. At t = 2 hours, the i.v. pentobarbital infusion was turned off and all animals were returned to their home cages and allowed to recover for chronic (4 week) neurological scoring. Ten additional animals were identically prepared but were not injured and served as sham controls.

Data Analysis:

Continuous variables compared across groups were examined utilizing analysis of variance (ANOVA) followed by Newman-Keuls tests. Continuous variables subjected to repeated measurements over time (e.g., cardiovascular measurements) were subjected to repeated measurement ANOVA followed by Dunnett's tests at each time point. Ordinal measurements such as neurological scores were evaluated utilizing the non-parametric Kruskal-Wallis ANOVA followed by individual non-parametric Mann-Whitney U-tests. Survival differences were compared using Fisher's Exact Probability Test. A 'p' value < 0.05 was considered statistically significant.

Cardiovascular Variables:

Low-injury (Group I) produced a transient (30 - 60 seconds) hypertensive response (from 100 ± 2 mm Hg to 131 ± 6 mm Hg), which returned to baseline levels by 5 minutes. A slight but gradual increase in MAP was observed in Group I animals over the remainder of the study period, and by 2 hours animals in Group I had become significantly hypertensive (mean = 109 ± 5 mm Hg) when compared to baseline ($p < 0.05$) and Group II or III animals ($p < 0.05$). Moderate-injury (Group II) produced a significant hypertensive response (from

96 ± 3 to 151 ± 4 mm Hg; $p < 0.05$), which returned to baseline by 5 minutes and remained stable thereafter. High-injury (Group III) produced a rapid and transient hypertensive response which increased from 107 ± 7 mm Hg to 170 ± 7 mm Hg at 30 seconds postinjury ($p < 0.05$) and fell to values significantly below baseline by 5 minutes postinjury (mean = 85 ± 6 mm Hg; $p < 0.05$). MAP remained significantly depressed when compared to baseline over the 2-hour period. No change in MAP in sham (uninjured) controls was observed over the study period.

Blood Gas Responses:

Animals injured at low (Group I) or moderate (Group II) severity demonstrated no significant changes in arterial blood gas (ABG) values over the course of the experiment. Animals injured at higher magnitude (Group III) demonstrated a transient apnea (mean = 30 seconds) immediately following the injury which was followed by a significant and transient decrease in pO_2 and increase in pCO_2 measured at 10 minutes postinjury. By 60 minutes postinjury, ABG values had returned to normal in all Group III animals. Sham (uninjured) controls had stable blood gas values over the 2-hour study period.

Electrophysiology:

Following low injury (Group I), compressed spectral edge from the injured and uninjured (contralateral) hemispheres fell by 5 minutes postinjury (from 12.0 ± 0.2 to 9.0 ± 0.7 Hz; $p < 0.05$) but returned to normal values by 10 minutes postinjury. EEG amplitude (total power) in the injured hemisphere fell significantly within the first 5 minutes following injury to 7% baseline ($p < 0.001$) and remained significantly depressed for the 2-hour study period. EEG amplitude in the uninjured hemisphere also fell significantly within the first 5 minutes to 24% of baseline ($p < 0.01$) but returned to normal by 1 hour following injury. No changes were observed in any individual EEG frequency (percentage or

absolute power of delta [0 - 3 Hz], theta [4 - 7 Hz], alpha [8 - 13 Hz] or beta [13 - 30 Hz] activity) analyzed by power spectral analysis.

Following injury of moderate severity (Group II), compressed spectral edge decreased within the first minute but rose to above baseline levels by 15 minutes postinjury. EEG amplitude (total power) in the injured hemisphere fell significantly within the first 5 minutes following injury to 4% of baseline ($p < 0.001$) and remained significantly depressed for the 2-hour study period (total amplitude at 2 hours = 44% of baseline; $p < 0.05$). EEG amplitude in the uninjured hemisphere also fell significantly but returned to 83% of baseline by 2 hours postinjury. A significant increase (91%) in delta (slow-wave, 1 - 3 Hz) activity was seen at 5 minutes following injury (from 14 ± 1 to 23 ± 2% of total power; $p < 0.05$). This relative increase in slow-wave activity gradually diminished over time and returned to baseline by 90 minutes postinjury. A 17% decrease in alpha (8 - 13 Hz; $p < 0.05$) and a 44% decrease in beta-wave activity (13 - 30 Hz; $p < 0.01$) was also observed within 5 minutes following brain injury of moderate severity. Alpha and beta EEG activity returned to normal by 1 hour postinjury.

Following brain injury of high severity (Group III), compressed spectral edge fell by 50% ($p < 0.001$) and remained significantly suppressed in the injured hemisphere over the duration of the study. EEG amplitude measured from the injured hemisphere fell markedly after injury, reached a nadir at 5 minutes (2% of baseline; $p < 0.001$) and remained significantly depressed up to 2 hours (28% of baseline, $p < 0.01$). EEG amplitude recorded from the contralateral (uninjured) hemisphere returned to normal levels by 1 hour postinjury. A significant increase (by 88%) in delta slow-wave, (1 - 3 Hz) activity occurred at 5 minutes following injury (17 ± 1 to 32 ± 3% of total power; $p < 0.01$) and

continued up to 90 minutes whereupon delta activity fell to baseline values. A 41% decrease in alpha activity ($p < 0.05$) and a 42% decrease in beta-wave activity ($p < 0.05$) occurred within 5 minutes following injury; alpha and beta EEG activity remained significantly suppressed over the 2 hour postinjury study period. Sham (uninjured) control animals showed no change in EEG recordings.

Brainstem Auditory Evoked Responses (BAERs):

BAERs were normal for all animals when recorded during the baseline period. Following low-grade injury (Group I), BAERs did not change with respect to peak latencies or wave amplitude. Following moderate injury (Group II), however, alterations in BAERs were observed. In 87% of animals (mean injury = 2.0 atm) receiving left ear stimulation, wave V disappeared at 1 hour postinjury. Peak latencies (I - III) were not significantly changed after injury, but peak latencies (I - IV) recorded from left ear stimulation were significantly prolonged following injury ($\bar{x}$ = 4 msec at 2 hours; $p < 0.05$), as were peak latencies I - IV ($\bar{x}$ = 3 msec at 2 hours; $p < 0.05$).

In Group III animals, wave V disappeared during right ear stimulation by 1 h postinjury. Peak latencies (I - III) were also significantly prolonged during right ear stimulation following injury ($\bar{x}$ = 4 msec at 2 hours; $p < 0.05$), as were peak latencies I - IV ($\bar{x}$ = 3 msec at 2 hours; $p < 0.05$).

Neurological Function:

Group I animals showed a minimal contralateral neurological deficit when tested at 8 and 24 hours. At postinjury weeks 1 and 2, 90% of Group I animals were still slightly neurologically impaired (median score = 15), but by 3 week, all animals had returned to preinjury neurological status. Group II animals showed a significantly greater neurological status. Group II animals showed a significantly greater neurological impairment than Group I when tested at 8 and

24 hours. By 1 week, all animals had moderate neurological deficit (median score = 10), which persisted for 4 weeks after injury. Animals injured at high level of injury (Group III) showed the worst overall impairment of neurological function. At 24 hours and 1 week postinjury, Group III animals showed a moderate neurological deficit. However, by week 2, virtually all animals demonstrated a severe neurological deficit that worsened over time.

Histopathological Studies:

Following low injury (Group I), minimal extravasation of Evans Blue Albumin (EBA) was observed in the left cortical hemisphere. After moderate injury (Group II) extravasation of EBA at 6 hours was maximal in the left frontoparietal cortex and external capsule and coincided with the distribution of maximal hemorrhage. After the very high levels of injury (Group III), both extravasation of EBA and hemorrhage extended radially from the left external capsule through the frontoparietal cortex, contacting the pial surface. The most severe vascular disruption was characterized by extensive hemorrhage and leakage of EBA into the frontoparietal cortex, along the course of the external capsule, within the left hippocampal gyrus, corpus collosum and the lateral ventricle. In all animals, subarachnoid hemorrhage was distributed over the left cerebral hemisphere and after high injury was also noted over the sulci of the cerebellum. Pathological examination performed at 1 month showed the development of frank cavitation in the left parietal (injured) cortex. Animals injured above 2.9 atm showed identical lesions at 24 hours often accompanied by necrotic changes in the cerebellum and small bilateral brainstem hemorrhage, reminiscent of the multiple petechial hemorrhages described by Adams.

Hematoxylin/eosin or toluidine blue stained brain sections revealed that at 24 hours after low-level injury (Group I), hemorrhage was localized to the left

cortical hemisphere and external capsule and extended approximately 3.5 - 4.0 mm, beginning slightly caudal to the level of the optic decussation. After low-level injury, hemorrhage typically occupied the external capsule bordering the lateral ventricle from approximately the level of the caudate-putamen to the level of the subthalamic nucleus.

After moderate impact (Group II) the pattern of hemorrhage was more extensive than that observed after low injury. Hemorrhage was present at the level of the optic decussation where it was primarily associated with the fimbria and external capsule and often associated with irruption of blood into the third ventricle. In two Group II animals, hemorrhage extended to the level of the pons in regions including the substantia nigra, external capsule, dentate gyrus and in a area just medial to the brachium of the inferior colliculus. In two animals, bilateral brainstem petechial hemorrhage was noted; in one case in the substantia nigra and in the second near the dentate gyrus. Hippocampal and cortical hemorrhage was common. In Group II animals, hemorrhage extended to the level of the pons in regions including the substantia nigra, external capsule, dentate gyrus and in an area just medial to the brachium of the inferior colliculus. In two animals, bilateral brainstem petechial hemorrhage was noted; in one case in the substantia nigra and in the second near the dentate gyrus. Hippocampal and cortical hemorrhage was common. In Group II animals, hemorrhage within the hippocampus was typically associated with region CA3. Cortical hemorrhage was noted within the frontoparietal, striate, and entorhinal cortex. Similar to that observed at 6 hours, petechial hemorrhage in the cortex was common and in areas of maximal hemorrhage, petechae extended from the pial surface inward toward the external capsule. In three animals, subarachnoid hemorrhage surrounded foci of intracortical coelescing petechial hemorrhages.

Survival:

All animals subjected to brain injury of light (Group I) and moderate (Group II) severity survived over the 4-week postinjury study period. Sixty percent of animals injured at a high level of severity (Group III) died within 1 week postinjury. These differences in mortality rates were found to be significant ($p < 0.05$).

Preliminary Nalmefene Data:

Animals (n = 4/group) were surgically prepared, injured and monitored as previously described. At 30 minutes following injury of moderate severity ($\bar{x}$ = 2.0 atm), animals were treated with either nalmefene hydrochloride (1.0 mg/kg, i.v., n = 4) or saline (equal volume; n = 4). Cardiovascular/electrophysiological monitoring continued for 2 hours postinjury and animals were returned to their home cages and allowed to recover for chronic (4-week) neurological scoring. At 24 hours postinjury, animals treated with nalmefene showed a trend toward improved electrophysiological and neurological status. Neurological function appeared to be improved at 1 and 2 weeks postinjury when compared to saline-treated controls. Further work must be performed to verify these results.

CONCLUSIONS

The present set of studies demonstrated that the somewhat κ-selective opiate antagonist WIN44,441-3 significantly improved mean arterial pressure, electroencephalographic activity, and localized cerebral blood flow following traumatic head injury in the cat. The beneficial effects of WIN44,441-3 were clearly stereospecific, since administration of its stereoisomer WIN44,441-2 had no effect on any physiological parameters following traumatic head injury. The observation that the beneficial effects of the WIN compound were stereospecific strongly support our hypothesis that the therapeutic efficacy of opiate antagonists in experimental head injury may be through actions at opiate receptors, possibly the κ-receptor. The beneficial effects of WIN(-) were found at doses substantially lower than those shown to be effective with naloxone, consistent with the hypothesis that the effects may be mediated through a κ-receptor mechanism of action. Our studies are the first to show an improvement in brain cerebral blood flow following administration of opiate antagonists in traumatic brain injury. This improvement of blood flow to specific brain regions such as midbrain, brainstem and basal ganglia following administration of WIN(-) suggests that opioid peptides released following head injury may act through opiate receptors to diminish cerebral blood flow. Our results employing WIN(-) in the cat were strengthened by the results we obtained utilizing nalmefene in the lateralized head injury model in the rat. Several pilot animals, treated with nalmefene (a newly synthesized, partially selective κ-opiate receptor antagonist), have demonstrated a recovery of mean arterial pressure and EEG variables following head injury. Traumatic brain injury in the rat was also found to be associated with time-dependent decreases in neurological function, which were directly correlated with the severity of injury. Our preliminary work with

the rat model has also suggested that somatosensory-evoked responses may not be as sensitive or predictive as brainstem auditory-evoked responses may not be as sensitive or predictive as brainstem auditory evoked responses following fluid-percussion head injury. Further work is currently under way to more fully evaluate the postinjury change in brainstem auditory-evoked responses that occur following fluid-percussion head injury in the rat. Additional studies will evaluate effects of receptor-selective opiate antagonists on neurologic outcome in rats. We also intend to examine the potential beneficial effects of physiologic opiate antagonists such as TRH on the rat model.

RECOMMENDATIONS

Our recent studies, combined with previous studies from our laboratory, provide the experimental basis for continued testing of selective opiate-receptor antagonists in traumatic head injury. Opiate antagonists such as WIN44,441-3, nalmefene or TRH analogs may provide a superior alternative to naloxone in the treatment of traumatic brain injury. In addition, we must continue to evaluate the physiological response to closed head injury in the rat model. Outcome measures in this model of lateralized head injury appear to be sensitive to severity of injury and pharmacological manipulation. Studies completed thus far suggest that the rat may serve as a reproducible and cost-effective model for the study of closed head injury. Further studies utilizing nalmefene and TRH or TRH analogs, should more clearly define the role of endogenous opioids in the pathophysiology of traumatic brain injury and may provide the basis for clinical trials with such compounds.

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